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Published in:
Microbial Drug Resistance

Link to article, DOI:
[10.1089/mdr.2010.0068](https://doi.org/10.1089/mdr.2010.0068)

Publication date:
2011

Document Version
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

Citation (APA):
Aarestrup, F. M., & Tvede, M. (2011). Susceptibility of *Clostridium difficile* Toward Antimicrobial Agents Used as Feed Additives for Food Animals. *Microbial Drug Resistance*, 17(1), 125-127.
<https://doi.org/10.1089/mdr.2010.0068>

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Susceptibility of *Clostridium difficile* Toward Antimicrobial Agents Used as Feed Additives for Food Animals

Frank M. Aarestrup¹ and Michael Tvede²

A total of 65 toxigenic *Clostridium difficile* strains isolated from patients with antibiotic-associated diarrhea were tested for susceptibility to avilamycin, flavomycin, monensin, and salinomycin. Except for flavomycin the substances showed *in vitro* efficacy comparable to reports of the currently most commonly used drugs for treatment of *C. difficile*. This indicates that these old compounds may be useful for the treatment of *C. difficile* infections in man and perhaps for other bacterial causes of diarrhea.

Introduction

CLOSTRIDIUM DIFFICILE IS AN ANAEROBIC spore-forming and often toxin-producing Gram-positive bacterium and is a major cause of pseudomembranous colitis and antibiotic-associated diarrhea mainly in patients previously administered antimicrobials, as first described by Larson *et al.*¹⁵ and Bartlett *et al.*⁶

C. difficile infections have recently increased in incidence and severity in many regions of North America and many countries in Europe.^{13,20} *C. difficile* is intrinsically resistant to many antimicrobial agents, and acquired resistance to the few therapeutically efficient drugs has been rapidly increasing.¹⁸ The current treatment regime is associated with treatment failure in some cases and relapses occur in ~20%–30% of all clinical cases.³ This has increased the interest in searching for novel and more efficient treatment options and the development of new antibiotics.^{5,13}

A number of antimicrobial agents, never approved for human therapy, have for several decades been used for nontherapeutic purposes or to control coccidia infections in food animals.^{1,2} Some of these compounds have shown good *in vitro* activity against *Clostridium perfringens*^{9,22} and have been also able to control infections with this bacterium in poultry^{19,21} and swine.¹⁴ These antimicrobials are included in the feed and are poorly absorbed in the gut. Thus, we speculated whether these compounds might have activity against *C. difficile*-caused gastrointestinal infections in humans.

This study was conducted to evaluate the *in vitro* susceptibility to avilamycin, flavomycin, monensin, and salinomycin against *C. difficile* isolated from infections in humans

as a first step to determine their potential value as human therapeutic drugs.

Materials and Methods

Bacterial isolates

A total of 65 clinical isolates of *C. difficile* were selected from cases of antibiotic-associated diarrhea at Rigshospitalet in Denmark. The isolates were cultured from feces on cycloserine–cefoxitin–fructose egg yolk agar (Statens Serum Institute) and identified as *C. difficile* by usual routine fermentation tests and demonstration of volatile fatty acids by gas–liquid chromatography according to the manual of the Virginia Polytechnic Institute.¹¹ Cytotoxin was demonstrated in a McCoy cell assay established in the laboratory at Rigshospitalet.¹²

Antimicrobial susceptibility testing

All isolates were examined for their minimum inhibitory concentrations (MICs) using twofold dilutions on *Brucella* agar supplemented with 5% laked sheep blood (Statens Serum Institute), 5 mg/L hemin (Sigma-Aldrich), and 1 mg/L vitamin K (Sigma-Aldrich) according to CLSI standards.⁸ Isolates were tested to the following concentrations of avilamycin (Elanco Animal Health), flavomycin (Hoechst GmbH), monensin (Elanco Animal Health), and salinomycin (Huvepharma): 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2, 4, 8, and 16 mg/L. Avilamycin and monensin were dissolved in acetone, salinomycin in 99% ethanol, and flavomycin directly in water before being added to the agar. As quality control strain, *C. difficile* ATTC 17857 was included on all agar plates.

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TABLE 1. SUSCEPTIBILITY OF 65 CLINICAL ISOLATES OF *CLOSTRIDIUM DIFFICILE* TOWARD FOUR ANTIMICROBIAL AGENTS USED AS FEED ADDITIVES OR AS COCCIDIOSTATICA IN FOOD ANIMALS

Antimicrobial agent	Number of isolates								
	0.03 ^a	0.06	0.13	0.25	0.5	1	2	4	8
Flavomycin	1			2	2	3	17	32 ^b	8
Monensin	2	7	4	7	29	16 ^b			
Salinomycin	8	4	15	20	18 ^b				
Avilamycin	1	9	23 ^b	30			2		

^aValues in this row indicate MICs (mg/L).

^bMIC of *Clostridium difficile* ATCC 17857.
MIC, minimum inhibitory concentration.

The agar plates were incubated under anaerobic conditions at 36°C for 42–48 hours.

Results

The MICs are shown in Table 1. MIC values for avilamycin ranged from ≤0.03 to 0.25 mg/L; two isolates, however, had MIC values of 2 mg/L. MIC values for flavomycin ranged from 0.25 to 8 mg/L, for monensin from ≤0.03 to 1 mg/L, and for salinomycin from ≤0.03 to 0.5 mg/L.

Discussion

Infections caused by *C. difficile* are increasing in prevalence and severity. Treatment of these infections is increasingly difficult because of the development of resistance toward fluoroquinolones and rifampicin and reduced clinical response to metronidazole and vancomycin. The use of vancomycin is further problematic because of the risk of selection for vancomycin-resistant enterococci and staphylococci.

We tested the *in vitro* susceptibility of 65 *C. difficile* isolates toward avilamycin, flavomycin, monensin, and salinomycin. All four compounds have been extensively used for decades in food animals and no toxicological reactions, to our knowledge, have been ever recorded. Avilamycin, an oligosaccharide, and another closely related antimicrobial agent, evernimicin, were previously under development for therapeutic use in humans. Avilamycin has been originally shown to bind to the 30S subunit of ribosome and thereby interfere with the protein synthesis function.²³ Evernimicin has, however, been shown to bind exclusively to the 50S subunit.¹⁷ In enterococci, resistance to both compounds is mediated by either point mutations in the L16 50S subunit ribosomal protein⁴ or a methyltransferase acting on the 50S subunit.¹⁶ Flavomycin is a phosphoglycopeptide that inhibits peptidoglycan synthesis by inhibiting peptidoglycan polymerases.⁷ Several bacterial species exhibit natural resistance to flavomycin, but so far acquired resistance has not been confirmed.⁷ Monensin and salinomycin are both ionophores and interfere with the natural ion transport system of both prokaryotic and eukaryotic cells. Staphylococcal and enterococcal isolates with reduced susceptibility has been reported,³ but the mechanism has never been elucidated. To our knowledge, no substances belonging to these classes have been ever developed for human usage.

All four substances tested had activity against *C. difficile*; however, flavomycin had relatively high MIC values,

whereas avilamycin, monensin, and salinomycin had MIC values comparable to or slightly lower than those reported for metronidazole and vancomycin.¹⁰

These results indicate that some of these old compounds, which for decades have been used for other purposes, might find application in human medicine for the treatment of *C. difficile* diarrhea and perhaps other chronic diarrhea diseases. Further studies are, however, necessary to determine the therapeutic potential and also to explore whether some negative effects might be associated with a future use of one or more of these compounds in human medicine.

Acknowledgments

The authors are grateful to Jacob Dyring Jensen and Anette Gregersen for technical assistance. This study was funded by a grant (274-05-0117) from the Danish Research Agency.

Disclosure Statement

No competing financial interests exist.

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